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Methamphetamine and HIV-1-induced neurotoxicity: Role of trace amine associated receptor 1 cAMP signaling in astrocytes

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Abstract

Methamphetamine (METH) is abused by about 5% of the United States population with approximately 10–15% of human immunodeficiency virus-1 (HIV-1) patients reporting its use. METH abuse accelerates the onset and severity of HIV-associated neurocognitive disorders (HAND) and astrocyte-induced neurotoxicity. METH activates G-protein coupled receptors such as trace amine associated receptor 1 (TAAR1) increasing intracellular cyclic adenosine monophosphate (cAMP) levels in presynaptic cells of monoaminergic systems. In the present study, we investigated the effects of METH and HIV-1 on primary human astrocyte TAAR1 expression, function and glutamate clearance. Our results demonstrate combined conditions increased TAAR1 mRNA levels 7-fold and increased intracellular cAMP levels. METH and beta-phenylethylamine (β -PEA), known TAAR1 agonists, increased intracellular cAMP levels in astrocytes. Further, TAAR1 knockdown significantly reduced intracellular cAMP levels in response to METH/ β -PEA, indicating signaling through astrocyte TAAR1. METH +/- HIV-1 decreased excitatory amino acid transporter-2 (EAAT-2) mRNA and significantly decreased glutamate clearance. RNA interference for TAAR1 prevented METH-mediated decreases in EAAT-2. TAAR1 knockdown significantly increased glutamate clearance, which was further heightened significantly by METH. Moreover, TAAR1 overexpression significantly decreased EAAT-2 levels and glutamate clearance that were further reduced by METH. Taken together, our data show that METH treatment activated TAAR1 leading to intracellular cAMP in human astrocytes and modulated glutamate clearance abilities. Furthermore, molecular alterations in astrocyte TAAR1 levels correspond to changes in astrocyte EAAT-2 levels and function. To our knowledge this is the first report implicating astrocyte TAAR1 as a novel receptor for METH during combined injury in the context of HAND.

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Keywords

methamphetamine; human immunodeficiency virus-1; astrocytes; trace amine associated receptor 1; cAMP

1. Introduction

Methamphetamine (METH) is an addictive pharmacological psychostimulant of the central nervous system (CNS). Ten percent of METH becomes biologically available within ten min of smoke inhalation, due to its high lipophilic nature (Volkow et al., 2010). METH generates an imbalance in the release and reuptake of dopamine, norepinephrine and epinephrine producing intense euphoria followed by hours of stimulation, excitation and alertness (Marshall and O'Dell, 2012). Risky behavior accompanies strong neurological impulses associated with METH abuse resulting in the high prevalence of METH users who acquire HIV-1 infection (Blackstone et al., 2013; Cisneros and Ghorpade, 2012). Further, clinical research describes that individuals infected with HIV-1 actively participate in METH abuse (Ellis et al., 2003; Harris et al., 1993; Semple et al., 2002).

HIV-1 infection often results in cognitive impairments, collectively termed HIV-associated neurocognitive disorders (HAND) (Lindl et al., 2010). Neurotoxic outcomes of METH abuse and HIV-1 CNS infection include, but are not limited to: brain hyperthermia, release of inflammatory mediators and reactive oxygen species (ROS), excitotoxicity, and astrogliosis (Cisneros and Ghorpade, 2012; Kiyatkin and Sharma, 2012; Rippeth et al., 2004); however, the molecular basis for these effects remains elusive. Additionally, as the most abundant cells of the CNS, astrocytes are a significant cell type affected by peripheral stimuli, such as METH and HIV-1.

Astrocytes function to support brain homeostasis and maintenance of the blood brain barrier (Sofroniew and Vinters, 2010). Astrocyte responses to external stimuli encompass increased intracellular cyclic adenosine monophosphate (cAMP) signaling and excitotoxicity (Miguel-Hidalgo, 2009; Volterra and Meldolesi, 2005). Astrocytes are responsible for clearing approximately 90% of extracellular glutamate from the synaptic cleft *via* excitatory amino acid transporter 2 (EAAT-2) (Anderson and Swanson, 2000). Additionally, cytokines, chemokines, biogenic and trace amines, neurotransmitters and pharmacological agents induce intracellular signaling cascades in astrocytes through activation of membrane and cytosolic receptors (Fraser et al., 1994; Glowinski et al., 1994; Kimelberg, 1995; Liu et al., 2004; Porter and McCarthy, 1997).

METH binds to cell membrane and intracellular receptors initiating rapid signaling events in astrocytes (Nagai and Yamada, 2010; Revel et al., 2011; Rodvelt and Miller, 2010; Schmitt and Reith, 2010; Zucchi et al., 2006). Trace amine associated receptor 1 (TAAR1) binds METH and regulates dopamine transporter trafficking in neurons (Miller et al., 2005). TAAR1 is a stimulatory G-protein coupled receptor (GPCR) activated by the metabolites of biogenic amines (Zucchi et al., 2006). Biological agonists for TAAR1 are trace amines, such as beta-phenylethylamine (β -PEA), octopamine and tryptamine (Zucchi et al., 2006). Activation results in intracellular cAMP signaling events, activation of protein kinase A

(PKA) and phosphorylation of downstream targets (Revel et al., 2011). METH exposure of astrocytes led to decreased EAAT-2 levels and function. Astrocyte cAMP signaling may regulate transcriptional, translational and post-translational modifications of EAAT-2. To our knowledge, expression and function of TAAR1 in astrocytes is not yet documented.

In this report, we investigated astrocyte TAAR1 in the presence of METH and HIV-1. We show METH +/- HIV-1 regulated EAAT-2 and TAAR1 expression and function. Further, METH and β -PEA exposure led to cAMP induction in astrocytes, which was prevented by TAAR1 RNA interference (RNAi). Finally, we demonstrate that a reduction in TAAR1 levels significantly increased, whereas, TAAR1 overexpression significantly decreased astrocyte glutamate clearance. Taken together, we propose that astrocyte responses to METH are mediated *via* TAAR1 activation and combined toxicity of METH and HIV-1 result in synergistic effects on astrocyte cAMP signaling and glutamate clearance abilities. We report for the first time a novel astrocyte receptor for METH-induced intracellular cAMP signaling.

2. Materials and Methods

2.1 Isolation, cultivation and activation of primary human astrocytes

Human astrocytes were isolated from first and early second trimester elected aborted specimens as previously described (Gardner et al., 2006). Briefly, tissues ranging from 82 to 127 days were procured in full compliance with the ethical guidelines of the National Institutes of Health, University of Washington and North Texas Health Science Center. Cell suspensions were centrifuged, washed, suspended in media, and plated at a density of 20×10^6 cells/150 cm². The adherent astrocytes were treated with trypsin and cultured under similar conditions to enhance the purity of replicating astroglial cells. These astrocyte preparations were routinely >99% pure as measured by immunocytochemistry staining for GFAP. Astrocytes were treated with METH (500 μ M, Sigma-Aldrich Inc., St. Louis, MO) and HIV-1 (p24, 10 ng/mL) alone and in combination at 37°C and 5% CO₂. The HIV-1_{ADA} isolate used was originally isolated from a patient with initials ADA and propagated *in vitro* as previously described (Gendelman et al., 1994). Dose- and time-kinetics revealed optimal concentrations for METH and HIV-1 were not toxic to astrocytes (data not shown).

2.2 RNA extraction and gene expression analyses

Astrocyte RNA was isolated as described in (Chadderton et al., 1997) 8 hr post-treatment and mRNA levels were assayed by real-time polymerase chain reaction (PCR). Taqman 5' nuclease real-time PCR was performed using StepOnePlus detection system and inventoried gene expression assays (Life Technologies Inc., Carlsbad, CA). Commercially available TaqMan® Gene Expression Assays were used to measure EAAT-2 (cat no. Hs00188189_m1), TAAR1 (cat no. Hs00373229_s1) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (cat no. 4310884E) mRNA levels. GAPDH, a ubiquitously expressed housekeeping gene, was used as an internal normalizing control. The 25 μ l reactions were carried out at 48°C for 30 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min in 96-well optical, real-time PCR plates. Transcripts were quantified by the comparative CT method, and represented as fold-change of control.

2.3 Glutamate clearance assay

Primary human astrocytes were plated in 48-well tissue culture plates at a density of 0.15×10^6 cells/well and allowed to recover for 24 hr prior to treatment as described in section 2.1. Following 24 hr treatment, glutamate (400 μ M) dissolved in phenol-free astrocyte medium was added into each well and clearance was assayed at 4 and 10 hr. The assay was performed and analyzed according to manufacturer's guidelines (Amplex Red Glutamic Acid/Glutamate Oxidase Assay Kit, Life Technologies).

2.4 Neurotoxicity and viability assays

Primary human astrocytes were plated in 48-well tissue culture plates at a density of 0.15×10^6 cells/well and allowed to recover for 24 hr prior to treatment as described in section 2.1. Following 24 hr treatment metabolic activity, lactate dehydrogenase release and apoptosis was measured. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, a colorimetric assay for measurement of metabolic activity, performed for cell viability (Manthorpe et al., 1986). The MTT assay was performed at appropriate time points, briefly five percent MTT reagent in astrocyte medium was added to astrocytes and incubated for 20–45 min at 37°C. The MTT solution was removed and crystals were dissolved in DMSO for 15 min with gentle agitation. The absorbance of the DMSO/crystal solution was assayed at 490 nm in a Spectromax M5 microplate reader (Molecular Devices, Sunnyvale, CA). Cytotoxicity by lactate dehydrogenase (LDH) release was quantified using the cytotoxicity detection kit (Roche Diagnostics, Indianapolis, IN, USA) according to manufacturer's instructions. DNA fragmentation and apoptosis was assayed using the double stranded DNA (dsDNA) ELISA (Roche Diagnostics, Indianapolis, IN, USA) according to the manufacturer's directions.

2.5 Immunocytochemistry

Astrocytes were cultured as adherent monolayers in 48-well plates at a density of 0.1×10^6 cells per well. Cells were fixed with ice cold acetone:methanol (1:1) for 30 min at -20°C and then blocked in phosphate buffered saline (PBS) with 2% bovine serum albumin (BSA) containing 0.1% Triton X-100 for 1 hr at room temperature. Cells were incubated with TAAR1 antibody (1:700, rabbit, Abcam, Cambridge, MA) and glial fibrillary acidic protein (GFAP) antibody (1:1000, chicken, Covance Inc., Emeryville, CA) in PBS (2% BSA, 0.1% Triton X) overnight at 4°C. Cultures were then washed and stained with Alexa Fluor® secondary antibodies (488 nm, green) and (594 nm, red) for 2 hr at room temperature (1:1000, Life Technologies). Nuclei were visualized with 4',6-diamidino-2-phenylindole (DAPI) (1:800, Life Technologies). Micrographs were obtained on a ECLIPSE Ti-300 using the NLS-Elements BR. 3.0 software (Nikon Inc., Melville, NY).

2.6 Confocal analysis

Human astrocytes were cultured on glass bottom 6-well tissue culture plates (MatTek Corp., Ashland, MA) at a density of 2×10^6 cells/well. The cells were carefully fixed with acetone:methanol (1:1) and stained with antibodies against TAAR1, GFAP, and DAPI at 24 hr post-treatment. Micrographs were obtained on an Olympus IX71 Microscope (Olympus America Inc., Center Valley, PA). Confocal colocalization analysis and two-dimensional

histogram was performed using BioimageXD software (Free Software Foundation Inc., Boston, MA).

2.7 cAMP assay

Intracellular cAMP levels in astrocytes were measured using a commercially available homogenous, bioluminescent assay, cAMP-Glo™ Assay, (Promega Corp., Madison, WI). Adherent monolayers of astrocytes cultured in 96-well plates (50,000 cells/well) were exposed to forskolin, METH and β -PEA (all from Sigma-Aldrich). Cells were directly activated and lysed in the tissue culture plate. Lysates were diluted to a final cell concentration of approximately 1,000 cells/ μ L in lysis buffer [500 μ M 3-isobutyl-1-methylxanthine (IBMX) and 100 μ M Ro 20-1724, cAMP specific phosphodiesterase inhibitors (both from Sigma-Aldrich)] and transferred to a white opaque flat bottom 96-well assay plates (Corning Inc. Life Sciences, Tewksbury, MA). Intracellular cAMP levels were assayed using GloMax 96 Microplate Luminometer with dual injectors (Promega). Data analysis for the half-maximal effective concentrations (EC₅₀) was performed with Prism V6.0 (GraphPad Software, La Jolla, CA) using sigmoidal dose-response (variable slope) curve.

2.8 Transfection of astrocytes

Cultured human astrocytes were transfected with On-Target plus® small interfering RNA (siRNA) pools specific to TAAR1 (siTAAR1), non-targeting control siRNA pools (siCON, Thermo Scientific, Waltham, MA, USA) and without siRNA (MOCK) or with overexpression plasmids CON-GFP or TAAR1-GFP (TrueORF cDNA Clones with C-term GFP tag, PrecisionShuttle Vector System, Origene Technologies Inc., Rockville, MD) using the Amaxa™ P3 primary cell 96-well Nucleofector kit and shuttle attachment (Lonza, Walkersville, MD, USA) according to the manufacturer's instructions. Briefly, 1.6 million astrocytes were suspended in 20 μ L nucleofector solution containing siCON or siTAAR1 (100 nM) or CON-GFP or TAAR1-GFP (0.25 μ g/1.6 million cells) and transfected using protocol CL133. Transfected cells were supplemented with astrocyte media and incubated for 30 min at 37°C prior to plating. Cells were allowed to recover for 48 hr prior to experimental use.

2.9 Statistical analyses

Statistical analyses were performed using Prism V6.0 with one-way analysis of variance (ANOVA) and Newman-Keuls post-test for multiple comparisons. Significance was set at $p < 0.05$ and data represents means $\pm$ standard error of the mean (SEM). Results are representative of two or more independent donors, denoted as n , with a minimum of three individual replicates in each experiment.

3. Results

3.1 METH and HIV-1 decreased astrocyte EAAT-2 levels and glutamate clearance

Glutamate clearance through astrocyte EAAT-2 is regulated by extracellular stimuli, including inflammatory mediators, pharmacological agents and bacterial/viral particles (Amara and Fontana, 2002; Colton et al., 2010; Gadea and Lopez-Colome, 2001). We

measured astrocyte EAAT-2 mRNA levels and their capacity to clear glutamate following 24 hr of METH and HIV-1, alone and in combination (Fig 1A and B). EAAT-2 mRNA levels decreased significantly with METH or HIV-1 alone ($*p<0.05$, $***p<0.001$, respectively) and by approximately 40% during combined conditions compared to control ($***p<0.001$, $n=5$). Similarly, astrocytes ability to clear extracellular glutamate 10 hr post-glutamate addition was significantly impaired subsequent to METH and/or HIV-1 treatments ($***p<0.001$, Fig 1B). GFAP is a recognized astrocyte marker responsive to injury, therefore we measured GFAP mRNA levels following METH and/or HIV-1 activation (Fig 1C). GFAP mRNA levels were not significantly different. In order to verify that changes in EAAT-2 were not a result of METH- and HIV-1-induced cytotoxicity, we measured astrocytes metabolic activity by MTT (Fig 1D), cytotoxicity by LDH release (Fig 1E), and apoptosis by dsDNA fragmentation ELISA (Fig 1F) following METH and/or HIV-1 treatment. No significant changes were observed in any parameters 24 hr post-stimulation (Fig 1D–F, respectively).

3.2 METH and HIV-1 regulate astrocyte TAAR1

To our knowledge, no previous evidence has been published demonstrating METH activation of astrocyte TAAR1; so we measured TAAR1 mRNA, protein levels and function in cultured human astrocytes following METH and/or HIV-1 treatment (Fig 2). Astrocyte TAAR1 mRNA levels did not change significantly with METH or HIV-1 alone; however, combined treatments significantly upregulated TAAR1 mRNA levels by approximately 7-fold (Fig 2A, $***p<0.001$, $n=3$). In neuronal cells TAAR1 activation results in intracellular cAMP increases (Nelson et al., 2007), thus we measured METH- and/or HIV-1-induced intracellular cAMP levels at 2 min and 10 min post-activation (Fig 2B). METH significantly increased cAMP levels within 2 min compared to control and continued to rise up to 10 min ($**p<0.01$). METH and/or HIV-1 significantly increased intracellular cAMP at 10 min in comparison to control ($**p<0.01$). In addition, 24 hr post-METH and/or HIV-1 treatment, astrocytes were immunostained for TAAR1 (Fig 2C, D, E and F). Cytoplasmic and nuclear localization was confirmed by confocal microscopy (Fig 2C1, D1, E1 and F1). Consistent with changes in RNA levels, combined treatments with METH and HIV-1 increased TAAR1 immunostaining (Fig 2A and F). Confocal microscopy (Fig 2C1, D1, E1 and F1) showed nuclear localization of TAAR1 (green) and perinuclear colocalization with GFAP (red). HIV-1 treatment alone or in combination with METH increased cytoplasmic and nuclear localization (Fig 2E1 and F1). Taken together, TAAR1 is expressed in astrocytes and modulated by combined treatments of METH and HIV-1.

3.3 Forskolin, METH and β -PEA increase astrocyte intracellular cAMP levels in a dose-dependent manner

Astrocytes were treated with multiple doses of forskolin, METH and β -PEA, followed by lysis and quantification of intracellular cAMP levels (Fig 3A). The half maximal effective concentration (EC_{50}) for forskolin, METH and β -PEA were measured in multiple biological astrocyte donors for intracellular cAMP levels. Representative dose response curves are shown with an EC_{50} of 10.96 μ M for forskolin (Fig 3A), 32.86 μ M for METH (Fig 3B) and 11.04 μ M for β -PEA (Fig 3C). When compared across multiple astrocyte donors the average EC_{50} was 12.88 ± 1.98 μ M for forskolin, 30.6 ± 5.05 μ M for METH and 11.01 ± 2.31 μ M

for β -PEA (Fig 3D). The efficacy for both METH and β -PEA was 40 nM cAMP; however, the lower EC₅₀ for β -PEA compared to METH indicates that METH has a lower potency compared to the known TAAR1 agonists, β -PEA.

3.4 RNAi for TAAR1 decreased astrocyte TAAR1 protein levels and cAMP responses

Astrocytes were MOCK-, siCON- and siTAAR1-transfected by nucleofection, plated for 48 hr and immunostained for TAAR1 following 24 hr of METH treatment. MOCK- and siCON-transfected astrocytes expressed TAAR1 protein levels as previously observed (Fig 4A and C). METH treatment did not increase TAAR1 protein levels in MOCK- or siCON-transfected astrocytes; however, astrocytes appeared reactive with greater colocalization of TAAR1 with GFAP (Figure 4B and D). siTAAR1 transfection decreased TAAR1 expression with or without METH (Fig 4E and F). Forskolin-, METH- and β -PEA-induced intracellular cAMP levels were quantified in MOCK-, siCON- and siTAAR1-transfected astrocytes and data at 500 μ M agonists concentration for each is shown (Fig 4G, n=4). Forskolin induction of cAMP was not significantly different across all treatments, averaging 44 nM (Fig 4G). METH-induced cAMP levels averaged 32.8 nM in MOCK-transfected astrocytes and 32.3 nM in siCON-transfected astrocytes. However, cAMP levels were approximately 88% lower in siTAAR1-transfected astrocytes, averaging 3.8 nM (Fig 4G, *** $p < 0.001$), indicating METH signaling *via* TAAR1. Likewise, β -PEA-mediated cAMP levels in siTAAR1-transfected astrocytes significantly decreased approximately 95% to an average of 1.6 nM when compared to the average cAMP of 29.3 nM in MOCK-transfected and 34 nM in siCON-transfected astrocytes (Fig 4G, *** $p < 0.001$).

3.5 TAAR1 RNAi and overexpression alter astrocyte glutamate clearance

Astrocytes were transfected as described in section 2.7. Cumulative data from two astrocyte donors tested in multiple replicates each is shown. METH significantly decreased EAAT-2 levels in MOCK- (* $p < 0.05$) and siCON-transfected (** $p < 0.01$) astrocytes (Fig 5A). In order to investigate the role of TAAR1 in astrocyte METH signaling we next employed TAAR1 RNAi. Downregulation of TAAR1 using siRNAs prevented METH-induced downregulation of EAAT-2 (Fig 5A). In parallel, glutamate uptake was significantly reduced in MOCK- (*** $p < 0.001$) and siCON-transfected (*** $p < 0.001$) astrocytes (Fig 5B). TAAR1 downregulation alone significantly increased glutamate clearance (*** $p < 0.001$) that was enhanced following METH treatment (Fig 5B, *** $p < 0.001$). To further study the relationship between TAAR1 and EAAT-2, we utilized a TAAR1-GFP overexpression plasmid. TAAR1 upregulation alone significantly decreased EAAT-2 levels (*** $p < 0.001$), which was exacerbated by METH treatment (Fig 5C, *** $p < 0.001$). Likewise, TAAR1 overexpression alone significantly impaired glutamate clearance abilities of astrocytes (*** $p < 0.001$) with little change following METH treatment (Fig 5D). Taken together, our data suggest TAAR1 mediates METH-induced EAAT-2 downregulation and impaired glutamate uptake by human astrocytes and may serve as a therapeutic target for the attenuation of excitotoxicity.

4. Discussion

We report for the first time a novel receptor that is activated following METH treatment in primary human astrocytes. In this study, we demonstrate that METH and HIV-1 regulated TAAR1 expression and cellular localization. TAAR1 agonists, METH and β -PEA, led to intracellular cAMP signaling that was interrupted with TAAR1 knockdown. METH and/or HIV-1 exposure decreased EAAT-2 expression and the ability of astrocytes to clear extracellular glutamate. TAAR1 downregulation prevented a reduction in EAAT-2 mRNA and increased glutamate clearance, which was enhanced upon METH treatment. In addition, TAAR1 overexpression alone reduced EAAT-2 levels that significantly decreased with METH and attenuated glutamate uptake in parallel. The mechanisms by which METH increases brain injury in the context of HIV-1 infection remain unclear; however, our studies reveal the effects of METH and HIV-1 on astrocyte-mediated excitotoxicity and direct mechanisms of METH-induced intracellular cAMP levels in astrocytes. Our data suggest TAAR1 mediates METH-induced EAAT-2 downregulation and impairs glutamate clearance thereby increasing the severity of HIV-1-induced excitotoxicity.

TAAR1 mediating METH-induced effects in astrocytes is a novel finding. TAAR1 is an intracellular GPCR involved in dopaminergic signaling (Barak et al., 2008; Bunzow et al., 2001; Lewin, 2006). TAAR1 plays a role in the etiology of various neurological disorders including schizophrenia (Revel et al., 2013). The neurobiology of schizophrenia is associated with imbalances in the dopaminergic and glutamatergic system (Gainetdinov et al., 2001). Further, the antipsychotic potential of TAAR1 agonists have been shown to be effective for the neurological therapy of both increased dopamine activity and reduced glutamatergic activity in schizophrenic models (Revel et al., 2013). We observed basal TAAR1 protein to be distributed both in the cytoplasm and nucleus of primary human astrocytes. HIV-1 alone or in combination with METH increased TAAR1 localization both in the cytoplasm and nucleus. Intracellular TAAR1 in eGFP-rhesus monkey transfected HEK293 cells was activated following agonist diffusion to the cytoplasm (Miller et al., 2005). While it is unclear how HIV-1 regulates TAAR1, HIV-1 relevant cytokines, including IL-1 β , increased TAAR1 levels in primary human astrocytes (data not shown). Moreover, in lymphocytes, TAAR1 levels increased following immune activation and METH-induced TAAR1 activation upregulated CREB (cAMP responsive element binding protein) and NFAT (nuclear factor of activated T-cell), transcription factors commonly associated with immune activation and EAAT-2 transcription (Panas et al., 2012). Nuclear localization of TAAR1 has not been previously reported; however, there is an increasing number of documented GPCRs in the nucleus (Boivin et al., 2008; Gobeil et al., 2006). Astrocyte TAAR1 nuclear activity is yet to be investigated.

Activation of TAAR1 leads to intracellular cAMP signaling that results in PKA and PKC phosphorylation and activation (Panas et al., 2012; Xie and Miller, 2007). Maximal efficacy for METH- and β -PEA-induced intracellular cAMP saturated at 40 nM suggesting they are full agonists for the target receptor. However, the average EC₅₀ for β -PEA was approximately 3-fold lower than the average METH EC₅₀ further implying that while both agonists produce similar efficacy, β -PEA has a higher potency for the receptor. Literature suggests that METH binds monoaminergic transporters/receptors and sigma receptors in

neurons, but the mechanisms in astrocytes are unknown (Revel et al., 2011). Interestingly, both METH and HIV-1, alone and in combination, significantly increased intracellular cAMP levels within 10 min of stimulation. Studies *in vitro* demonstrated that HIV-1 infection of primary T cells increased intracellular cAMP levels (Moreno-Fernandez et al., 2012; Nokta and Pollard, 1991; Potula et al., 2010) and lymphocytes from HIV-1-infected patients had two-fold higher intracellular cAMP levels than lymphocytes from uninfected individuals (Aandahl et al., 1998; Hofmann et al., 1993).

The canonical cAMP pathway involves PKA activation. Activation of both PKA and PKC have been reported downstream of METH-induced TAAR1 cAMP signaling in neurons (Miller et al., 2005; Panas et al., 2012). These pathways regulate genes involved in cell cycle, cell survival and cytokine secretion (Paramanik and Thakur, 2013). Downstream activation of PKA and PKC leads to the modulation of regulatory units on the EAAT-2 promoter, translational and post-translational modifications and ubiquitination (Casado et al., 1993; Kalandadze et al., 2002; Martinez-Villarreal et al., 2012; Schlag et al., 1998; Tan et al., 1999). Analysis of the EAAT-2 promoter showed that NF- κ B is an important regulator of EAAT-2 transcription in astrocytes (Aida et al., 2011) and the rate of NF- κ B translocation into the nucleus is regulated by the activation of PKA, downstream of cAMP (King et al., 2011). METH may be regulating EAAT-2 transcription *via* downstream signaling pathways associated with TAAR1 activation; however, previous reports suggest that intracellular activation of cAMP and PKA lead to increased EAAT-2 transcription in astrocytes (Karki et al., 2013; Kim et al., 2011). Others report that cAMP signaling often fails to induce EAAT-2 transcription in glioma cell lines, proposing that intrinsic factors, such as DNA methylation, keep the EAAT-2 gene inactive and unresponsive to external stimuli (Yang et al., 2010; Zschocke et al., 2007). Additionally, there are three forms of human EAAT-2 transcripts, two are not constitutively translated, requiring extracellular factors to induce translation (Colton et al., 2010). EAAT-2 expression is regulated at both the transcriptional and translational levels. The efficiency of EAAT-2 translation suggests that EAAT-2 regulation occurs predominately at the translational and post-translational levels (Tian et al., 2007).

In vitro, HIV-1 results in EAAT-2 downregulation and reduced glutamate uptake in astrocytes (Wang et al., 2003). While METH is known to induce neuronal excitotoxicity, the direct mechanisms of METH-induced EAAT-2 dysregulation in astrocytes remain unclear. EAAT-2 activity is regulated through gene expression, protein targeting and trafficking and post-translational modifications. METH may be modulating EAAT-2 *via* downstream signaling of TAAR1. An increase in intracellular cAMP levels result in astrocyte glutamate release (Gochenauer and Robinson, 2001). Furthermore, signaling pathways involving PKC and PKA are differentially responsible for early and late phase increases of astrocyte glutamate uptake, EAAT-2 trafficking and degradation (Pita-Almenar et al., 2006). Therefore, the robust changes observed in astrocyte glutamate uptake following METH treatment maybe mediated by translation and trafficking of EAAT-2. Further, selective TAAR1 activation in brain slices from mice blocked activity of NMDA antagonists, which was not observed in TAAR1 knockout mice, suggesting TAAR1 involvement in glutamatergic signaling (Revel et al., 2011). Basal TAAR1 levels and the robust changes in

glutamate clearance following TAAR1 knockdown and overexpression suggest it may have broader implications in astrocyte function.

Astrocytes have not been previously shown to express receptors that are directly sensitive to METH. We show for the first time METH-mediated activation of astrocyte TAAR1 and its implication in EAAT-2 regulation. Since EAAT-2 accounts for the majority of glutamate uptake from the synaptic cleft, METH-mediated changes in EAAT-2 transcription and activity may result in additive and/or synergistic effects associated with HIV-1-induced excitotoxicity. Our results are intriguing and indicate that METH-induced TAAR1 activation in human astrocytes is a novel pathway leading to astrocyte EAAT-2 regulation in the context of HIV-associated neurocognitive disorders and may have future therapeutic implications.

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Abbreviations

β-PEA	beta-phenylethylamine
cAMP	cyclic adenosine monophosphate
CON-GFP	control-green fluorescent protein
EAAT-2	excitatory amino acid transporter-2
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GFAP	glial fibrillary acidic protein
GPCR	G-protein coupled receptor
HIV-1	human immunodeficiency virus-1
HAND	HIV-associated neurocognitive disorders
LDH	lactate dehydrogenase
METH	methamphetamine
PKA	protein kinase A
ROS	reactive oxygen species
TAAR1	trace amine associated receptor 1
TAAR1-GFP	trace amine associated receptor 1-green fluorescent protein

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Highlights

- Primary human astrocytes express functional TAAR1
- TAAR1 agonists, METH and β -PEA, lead to increased intracellular cAMP levels in primary human astrocytes
- TAAR1 is a potential receptor for METH-associated effects in human astrocytes
- METH and HIV-1 modulate astrocyte glutamate clearance abilities

Figure 1. METH and HIV-1 modulate excitotoxicity and astrocyte viability

Astrocytes were treated with METH (500 μ M) and/or HIV-1 (p24 10 ng/mL). RNA was isolated at 8 hr and assayed for EAAT-2 levels by real-time PCR. EAAT-2 levels were significantly lower in astrocytes treated with METH (* $p < 0.05$), HIV-1 (*** $p < 0.001$) and METH + HIV-1 (A, *** $p < 0.001$, $n=5$). Glutamate clearance was measured at 4 hr and 10 hr post-glutamate addition and was significantly decreased with METH, HIV-1 and in combined conditions at 10 hr (B, *** $p < 0.001$, $n=3$). No significant differences in GFAP mRNA levels were observed (C). To determine astrocyte viability, metabolic activity (D), cytotoxicity (E) and apoptosis (F) were measured following 24 hr activation with METH and/or HIV-1. Transient treatment of METH and/or HIV-1 did not significantly increase MTT, LDH activity or apoptosis (D–F, $n=3$). Multiple astrocyte donors (n) were tested, each analyzed in a minimum of triplicate determinations.

Figure 2. Astrocyte TAAR1 expression and localization is regulated by METH and HIV-1
Human astrocytes were treated with METH (500 μ M) and/or HIV-1 (p24 10 ng/mL) for 8 hr and RNA was isolated and assayed for TAAR1. TAAR1 mRNA levels were significantly increased with METH + HIV-1 cotreatments (A, *** $p < 0.001$, $n=3$, independent astrocyte donors). METH-and/or HIV-1-induced intracellular cAMP was measured at 2 min and 10 min post-activation (B). METH alone significantly induced intracellular cAMP at both time points (** $p < 0.01$). HIV-1 alone and in combination with METH significantly induced intracellular cAMP at 10 min post-activation (** $p < 0.01$). Astrocytes were fixed and immunostained for GFAP (red) and TAAR1 (green). TAAR1 expression was localized in both cytoplasm and nucleus (C, D, E and F). To further confirm nuclear localization, confocal microscopy was performed (C1, D1, E1 and F1). Activation with HIV-1 alone (E1) and in combination with METH (F1) increased nuclear TAAR1 localization.

Figure 3. Forskolin, METH and β -PEA induce astrocyte intracellular cAMP signaling
Astrocyte intracellular cAMP signaling was induced with forskolin (A) as a positive control, METH (B) and β -PEA (C), known TAAR1 agonists. Representative donor EC_{50} values for forskolin (10.96 μM), METH (32.86 μM), and β -PEA (11.04 μM) are shown. Intracellular cAMP induction assays were determined for multiple biological donors (n), and cumulative data is illustrated in panel D.

Figure 4. RNA interference downregulates astrocyte TAAR1 protein levels and intracellular cAMP in response to METH and β -PEA

Human astrocytes were MOCK-, siCON- and siTAAR1-transfected and plated for 48 hr followed by 24 hr of METH (500 μ M) treatment. Cells were fixed and probed for TAAR1 (green) and GFAP (red). MOCK- (A) and siCON- (C) transfected astrocytes showed TAAR1 protein expression with and without METH treatment at 24 hr. METH (500 μ M) treated MOCK- and siCON-transfected astrocytes demonstrated similar levels of TAAR1 protein (B and D). As expected siTAAR1-transfected astrocytes demonstrated reduced TAAR1 expression (E and F). Intracellular cAMP levels were quantified in MOCK-, siCON- and siTAAR1-transfected astrocytes. siTAAR1-transfected astrocytes showed

significantly reduced METH and β -PEA-induced intracellular cAMP (**G**, *** $p < 0.001$, n=4, independent astrocyte donors).

Figure 5. METH-induced TAAR1 activation regulates EAAT-2 mRNA levels and function
Primary human astrocytes were MOCK-, siCON-, siTAAR1-, CON-GFP- or TAAR1-GFP-transfected and treated with METH (500 μ M). Two independent astrocyte donors were tested with a minimum of triplicate determinations in each experiment. Data was analyzed as fold change to internal control and presented as cumulative data for both donors. RNA was collected at 8 hr and glutamate clearance was measured at 24 hr post-treatment. **(A)** EAAT-2 mRNA levels significantly decreased in MOCK- and siCON-transfected astrocytes treated with METH (* $p < 0.05$, ** $p < 0.01$). Changes in METH-mediated EAAT-2 levels were blocked by siTAAR1 transfection. **(B)** Glutamate clearance was performed in parallel. METH treatment significantly decreased glutamate clearance in MOCK- and siCON-transfected astrocytes while siTAAR1 transfection significantly increased glutamate clearance compared to METH treated controls (*** $p < 0.001$). **(C & D)** Both TAAR1-GFP-transfection and METH treatment significantly lowered levels of EAAT-2 mRNA expression in astrocytes and in parallel resulted in an exacerbated reduction in glutamate clearance abilities (*** $p < 0.001$).